FEBS 18209 FEBS Letters 405 (1997) 1–4

Detection of glycosyl-phosphatidylinositol-anchored proteins on the surface of *Nicotiana tabacum* protoplasts

Adam M. Takos[a], Ian B. Dry[b], Kathleen L. Soole[a,*]

[a]*Centre for Plant Membrane Biology, School of Biological Sciences, The Flinders University of South Australia, GPO Box 2100, Adelaide, SA 5001, Australia*
[b]*CSIRO Division of Horticulture, Hartley Grove, Urrbrae, SA 5064, Australia*

Received 2 January 1997

Abstract Glycosyl-phosphatidylinositol (GPI)-anchored plasma membrane proteins have been found to be widespread in eukaryotes and protozoa but have not been reported in higher terrestrial plants. A sensitive biotin-based assay has been used to detect the presence of GPI-anchored proteins on the outer surface of cultured *Nicotiana tabacum* cells. Six proteins with molecular weights of 92, 84, 60.5, 54.5, 39.5 and 37 kDa were found to move from a Triton X-114 detergent-rich phase to an aqueous phase following incubation with phosphatidylinositol-specific phospholipase C (PtdIns-PLC). The behaviour of these proteins is consistent with the presence of a GPI-anchor. Seven GPI-anchored proteins were also detected on the surface of tobacco leaf protoplasts with molecular weights of 67.5, 62, 39, 33.5, 27, 23 and 15.6 kDa. These data demonstrate the presence of multiple GPI-anchored proteins on the plasma membrane of higher plant cells.

Key words: Plant; GPI-anchored; Biotin; Plasma membrane

1. Introduction

Cell surface proteins carry out a number of important functions including cell-cell recognition, cell adhesion, ion transport and signal transduction. While many of these proteins are attached via one or multiple hydrophobic transmembrane domains, it has become recognised more recently that a significant number of cell surface proteins are anchored to the membrane by a lipid anchor [1]. The most common lipid anchor is glycosyl-phosphatidylinositol (GPI) which consists of a glycan linkage containing ethanolamine linked to the C-terminal of the protein, three mannose residues and glucosamine linked to the inositol group of phosphatidylinositol which is embedded in the plasma membrane (Fig. 1). The hydrophobic component of this structure has been shown to be susceptible to cleavage from the protein by phosphatidylinositol-specific phospholipase C (PtdIns-PLC) [3]. A diverse array of membrane proteins has been identified as GPI-anchored including hydrolytic enzymes, mammalian and protozoal antigens and cell adhesion molecules [4]. It is not clear as to what function or property a GPI-anchor may confer upon membrane proteins although several possibilities include prolonging the cell surface half life, increased mobility in the plane of the lipid bilayer, regulated release from the cell surface by endogenous phospholipases, transduction of extracellular signals, dominant targeting to a specific membrane domain and improved ability for intercepting extracellular substrates [5,6].

Since the elucidation of the GPI-anchor structure 10 years ago, this class of proteins has been found to be present in a wide range of mammalian cell types, yeast and protozoa, but had not been reported in plants. Recently Stöhr et al. [7] reported the presence of a GPI-anchored nitrate reductase in the green algae *Chlorella saccharophila*. Plasma membrane-bound nitrate reductase was found to be labelled with [^{3}H]ethanolamine, a component of the anchor structure (Fig. 1) and could be released from the cell surface by PtdIns-PLC treatment, providing conclusive evidence for GPI-anchored proteins in algae. More recently, Morita et al. [8] have suggested that alkaline phosphatase from the aquatic monocot *Spirodela oligorrhiza* may be GPI-anchored. However, while it could be shown that alkaline phosphatase was labelled with [^{3}H]ethanolamine, this form of alkaline phosphatase was not sensitive to PtdIns-PLC cleavage. Also a large proportion of the enzyme was hydrophilic in nature and not associated with a lipid anchor structure. The aim of our study, therefore, has been to confirm the presence of GPI-anchored proteins in higher plant cells. Using a sensitive biotin-based assay, we have found evidence of multiple GPI-anchored proteins on the plasma membrane of *Nicotiana tabacum* cells.

2. Materials and methods

2.1. Materials

All biochemicals including Murashige and Skoog (MS) basal salt mix, *Bacillus cereus* PtdIns-PLC, Streptavidin-horse radish peroxidase (POD) conjugate and protease inhibitors were purchased from Sigma (St. Louis, MO) or Boehringer Mannheim Australia (Castle Hill, NSW). Cellulase (Onozuka) RS was obtained from Yakult Pharmaceutical (Tokyo, Japan) and Pectolyase Y-23 from Seishin Corp. (Tokyo, Japan). Immunopure sulfosuccinimidyl-6-(biotinamido) hexanoate (NHS-LC-biotin) and chemiluminescent horse radish peroxidase substrate system were obtained from Pierce (Rockford, IL).

2.2. Plant cell culture

Tobacco plants were grown under natural light in a glasshouse at 25°C. *N. tabacum* (NT-1) cells were maintained in suspension culture by subculturing 1:25 every 7 days in cell culture medium (CCM) described in [9] and incubated at 25°C on an orbital shaker at 100 rpm in continuous light. For protoplast isolation, cells were subcultured 1:10 in 50 ml of CCM and grown for 3-5 days.

2.3. Protoplast isolation

NT-1 cells were pelleted in a swing-out rotor for 5 min at 100×*g*. The supernatant was discarded and the pellet washed twice in 50 ml of washing solution (0.4 M mannitol, 20 mM Mes, pH 5.8). Cells were

*Corresponding author: Fax: (+61) 88-201-3015.
E-mail: bikls@gamgee.cc.flinders.edu.au

Abbreviations: GPI, glycosyl-phosphatidylinositol; TX-114, octylphenoxypolyethoxyethanol; PtdIns-PLC, phosphatidylinositol-specific phospholipase C; NHS-LC-biotin, sulfosuccinimidyl-6-(biotinamido) hexanoate

PII S0014-5793(97)00064-1

incubated in protoplast enzyme solution (1.0%, w/v) Cellulase RS, 0.1% (w/v) pectolyase Y-23, 0.4 M mannitol, 20 mM Mes, pH 5.8) at 1-1.5 ml g^{-1} wet weight of cells, at 25°C on an orbital shaker at 40-50 rpm for 1-2 h. Alternatively protoplasts were isolated from 8-10 cm leaves harvested from 6-8-week-old plants as described [10].

2.4. Cell surface biotin labelling

Protoplasts (isolated from 50 ml of cell suspension culture or 30 g of leaves) were resuspended in 50 ml of washing solution and pelleted in a swing-out rotor for 5 min at $100\times g$. The pellet was washed twice in 50 ml of biotin labelling solution (10 mM triethanolamine, 137 mM NaCl, 3 mM KCl, 0.4 M mannitol, pH 9.0) and then left resuspended in the same solution to a volume of 5 ml. NHS-LC-biotin was added (0.5 mg ml^{-1} final) and the protoplasts incubated for 30 min with gentle agitation. Protoplasts were then washed again in biotin labelling solution and the incubation repeated with fresh NHS-LC-biotin. Biotin blocking solution (50 mM glycine, 0.4 M mannitol, in phosphate-buffered saline (PBS, pH 7.5) was added to a final volume of 50 ml, cells pelleted as above and left resuspended in 10 ml of biotin blocking solution for 10 min with gentle agitation. Washing and incubation in biotin blocking solution was repeated twice. All solutions used were ice cold and all centrifugations and incubations carried out at 4°C.

2.5. Cell lysis, phase separation and PtdIns-PLC treatment

Biotin-labelled protoplasts (suspended in ~4 ml of biotin blocking solution) at a concentration ranging from $6\text{-}20\times10^6$ cells ml^{-1}, were decanted into microfuge tubes and spun at $100\times g$ at 4°C for 5 min. The supernatants were discarded and each pellet resuspended in 1.5 ml of TX-114 lysis buffer (1%, v/v) TX-114 in Tris-buffered saline (TBS, pH 7.5), incubated on ice for 30 min and then lysed by vigorous vortexing. The lysate was clarified by centrifugation twice at maximum speed in a microfuge at 4°C for 10 min. Phase separation and *B. thuringiensis* PtdIns-PLC treatment was carried out essentially as described in [11]. In some experiments PtdIns-PLC from *B. cereus* was used at 1.2 U ml^{-1} in an incubation buffer containing 70 mM triethanolamine, 0.16% (w/v) sodium deoxycholate, 0.25 M methyl-α-D-mannopyranoside, pH 7.5. For both sources of PtdIns-PLC, treatment was for 4 h at 37°C with frequent mixing. Final aqueous phases were treated with 300 μl of a 75% (w/v) slurry of phenyl-Sepharose in TBS for 16-20 h on a rotating wheel at 4°C. Phenyl-Sepharose was removed by three sequential centrifugations at maximum speed in a microfuge for 1 min at 4°C. Solutions used for protoplasts isolation, cell lysis, phase separation and PtdIns-PLC treatment contained protease inhibitors leupeptin (20 μg ml^{-1}), pepstatin A (10 μg ml^{-1}), antipain (10 μg ml^{-1}) and PMSF (0.1 mM).

2.6. Detection of biotinylated proteins

Proteins in final aqueous and detergent phases were precipitated with 6% (w/v) trichloroacetic acid and 132 μg ml^{-1} sodium deoxycholate [12]. After addition of equal volumes of Laemmli sample buffer [13], excess acid was neutralised with NH_4OH vapours and samples boiled at 95°C for 5 min. Samples were then subjected to SDS-PAGE under reducing conditions on 4-20% gradient slab Tris-glycine gels. Proteins were transferred to an Immobilon PVDF membrane by dry blot. Blots were blocked overnight at 4°C with 3% (w/v) BSA, 1% (w/v) non-fat dry milk in PBS containing 0.5% (v/v) Tween 20, 10% (v/v) glycerol, 1 M glucose (PBS-TGG) [14]. After rinsing, the membrane in PBS containing 0.5% Tween 20 (PBST), the membrane was probed with a Streptavidin-POD conjugate (500 U conjugate ml^{-1}) diluted 1:5000 in PBS-TGG containing 0.3% (w/v) BSA for 2 h at room temperature on a rocking platform. Proteins were then detected by chemiluminesence on X-ray film. Densitometry analysis and molecular weight calculation was performed on computer-scanned images of blots using ImageQuant and Fragment Analysis for Windows NT software from Molecular Dynamics (Sunnyvale, CA).

3. Results

Lisanti et al. [15] previously developed a method to detect GPI-anchored proteins in polarized epithelial cells which exploits three conserved features of this class of proteins: (i) presence solely on the ectoplasmic leaflet of the lipid bilayer, (ii) susceptibility of the GPI-anchor to cleavage by PtdIns-PLC and (iii) PtdIns-PLC-induced transition of the protein from a hydrophobic to a hydrophilic form due to the loss of diacylglycerol from the anchor structure [15]. The phase partition assay utilises biotin, a membrane impermeable marker to selectively label cell surface proteins, followed by separation of hydrophobic and hydrophilic proteins by temperature-induced phase separation of the detergent Triton X-114 [16]. Treatment of the resultant detergent phase (containing integral and GPI-anchored proteins) with PtdIns-PLC and a subsequent second phase separation results in the identification of GPI-anchored proteins which move from the detergent to the aqueous phase [11,17].

The results of a phase partition assay conducted on protoplasts isolated from NT-1 suspension cells are shown in Fig. 2. Similar results were obtained over five separate experiments. Six proteins were found to move from the hydrophobic detergent phase to the aqueous phase following PtdIns-PLC treatment and phase separation (Fig. 2, lane 2). The approximate molecular weights of these proteins, based on molecular weight standards (Fig. 2, lanes 3 and 4), were 92, 84, 60.5, 54.5, 39.5 and 37 kDa. No proteins were detected in the aqueous phase when the detergent phase was incubated in the absence of PtdIns-PLC (Fig. 2, lane 5). This conclusively demonstrates that the appearance of proteins in the PtdIns-PLC-treated lane is the result of phospholipase-mediated cleavage of GPI-anchored hydrophilic membrane proteins

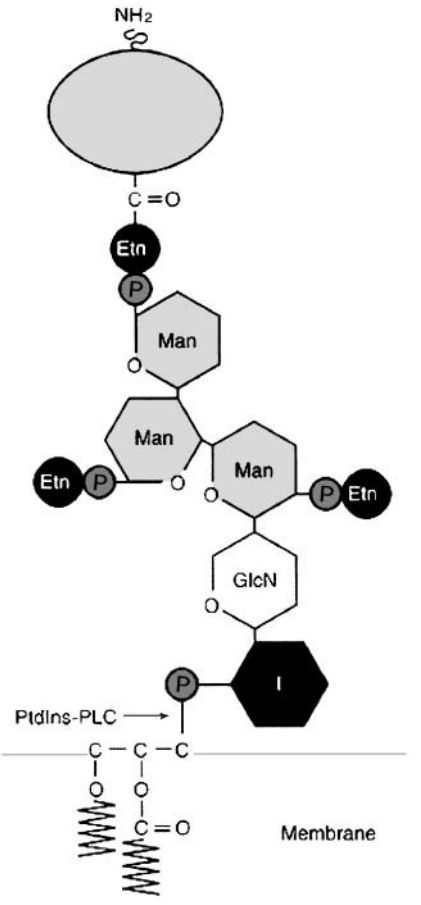

Fig. 1. Structure of a typical GPI-anchored protein. I, inositol; GlcN, glucosamine; Man, mannose; Etn, ethanolamine; P, phosphate. An arrow indicates the bond cleavable by PtdIns-PLC. The figure was adapted from [2].

and not the result of proteolytic activity or the solubilisation of hydrophobic proteins in the presence of traces of detergent. It is also noticeable that two bands at 39.5 and 37 kDa (arrows) are less intense in the detergent phase resulting from PtdIns-PLC treatment (Fig. 2, lane 1) than in the detergent phase incubated in the absence of PtdIns-PLC (Fig. 2, lane 6). This indicates the shift of these proteins to the aqueous phase upon PtdIns-PLC treatment and retention in the detergent phase in the absence of PtdIns-PLC.

It has recently been shown that exogenous GPI-anchored proteins may be able to spontaneously incorporate into the lipid bilayer [18]. In order to eliminate the possibility that the protoplasting enzymes that were used in these experiments were a source of contaminating GPI-anchored proteins, the assay was repeated with undigested NT-1 cells. In densitometry scans of the aqueous phases resulting from PtdIns-PLC treatment that the six peaks corresponding to GPI-anchored proteins in NT-1 protoplasts (Fig. 3A) are also found in undigested cells (Fig. 3B) at similar molecular weights. Thus, contamination of protoplasts with exogenous GPI-anchored proteins from the protoplasting solution can be dismissed. An additional minor seventh peak at 30 kDa is also evident in undigested cells and may represent a GPI-anchored protein that is somehow cleaved during protoplast isolation as it was not observed in any of the NT-1 protoplast experiments. In general, the yield of biotin-labelled GPI-anchored proteins appear smaller with undigested cells than with protoplasts. This may be due to a lower efficiency of biotin labelling in the presence of the cell wall.

Having successfully detected GPI-anchored proteins in suspension cells, the phase partition assay was used on protoplasts isolated from tobacco leaf tissue (Fig. 4). At least seven proteins were detected which moved from the detergent phase to the aqueous phase after incubation with PtdIns-PLC (Fig. 4, lane 2). The approximate molecular weights of these proteins, based on molecular weight standards (Fig. 4, lane 3), were 67.5, 62, 39, 33.5, 27, 23 and 15.6 kDa. The four largest molecular weight proteins are of similar size to proteins detected in NT-1 cultured cells (Fig. 2, lane 2) and could possibly be the analogous proteins in leaf tissue. There is a small degree of background in the absence of PtdIns-PLC treatment (Fig. 4, lane 4) at approximately 38 kDa and this could be the result of low level of an endogenous PtdIns-PLC or protease activity. However, it is clear that the majority of proteins detected in the aqueous phase (Fig. 4, lane 2) are the specific result of PtdIns-PLC digestion as the degree of background is small in comparison (Fig. 4, lane 4).

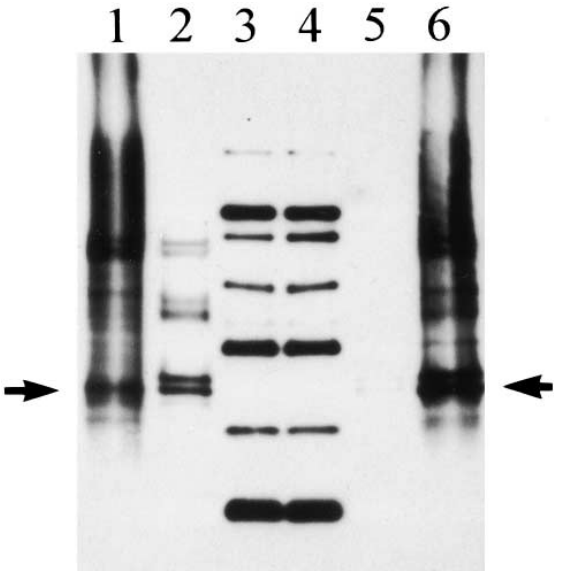

Fig. 2. Detection of GPI-anchored proteins on the surface of NT-1 protoplasts by phase partition assay. Protoplasts derived from NT-1 cells were labelled with biotin and hydrophobic proteins concentrated in a TX-114 detergent phase by phase partioning. This detergent phase was incubated in the presence or absence of PtdIns-PLC and proteins separated into detergent and aqueous phases by a second round of phase partioning. Biotinylated proteins were detected by Western analysis. Lane 1 (detergent phase) and lane 2 (aqueous phase) show protein partioning after PtdIns-PLC treatment; lane 5 (aqueous phase) and lane 6 (detergent phase) show protein partioning without PtdIns-PLC treatment. Lanes 3 and 4 show molecular weight standards: 200000, 116250, 97400, 66200, 45000, 31000, 21500, 14400 and 6500 Da.

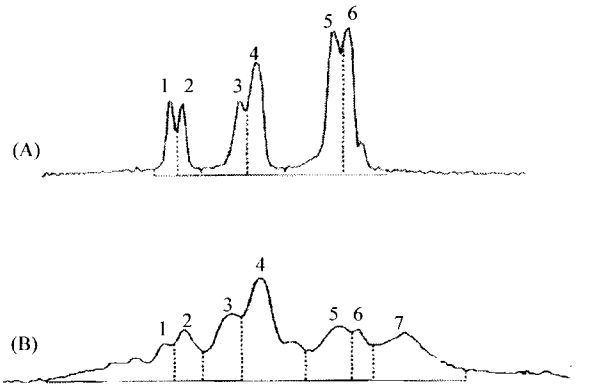

Fig. 3. Densitometry scans of Western blots of aqueous phases resulting from incubation with PtdIns-PLC. A: NT-1 protoplasts (scan of Fig. 2, lane 2). B: Undigested NT-1 cells.

4. Discussion

In comparison to the membrane proteins of chloroplast and mitochondria, sophisticated molecular biology techniques have been applied less frequently to the study of plasma membrane proteins in plant cells [19]. The phase transfer assay we have employed in this study specifically detects GPI-anchored proteins by the action of PtdIns-PLC, which cleaves the hydrophobic anchor domain, thus converting only GPI-anchored proteins to a hydrophilic form. In combination with the biotin-Streptavidin detection system, this is an extremely sensitive method of detection of GPI-anchored proteins [11]. Using this method six proteins have been detected on the plasma membrane surface of *N. tabacum* suspension cells which display properties consistent with the presence of a GPI-anchor. A seventh protein identified with whole cells (Fig. 3B) may be lost during protoplast isolation. Seven proteins, some of similar molecular weight to those in suspension cells, were also detected on the surface of *N. tabacum* leaf tissue (Fig. 4). We believe this represents the first evidence for GPI-anchored proteins associated with the ectoplasmic face of the plasma membrane of higher plant cells.

Recent studies have provided evidence for GPI-anchored alkaline phosphatase in the aquatic monocot *S. oligorrhiza* [8] and nitrate reductase in the algae *C. saccharophila* [7]. In both cases, [^{3}H]ethanolamine labelling did not reveal any oth-

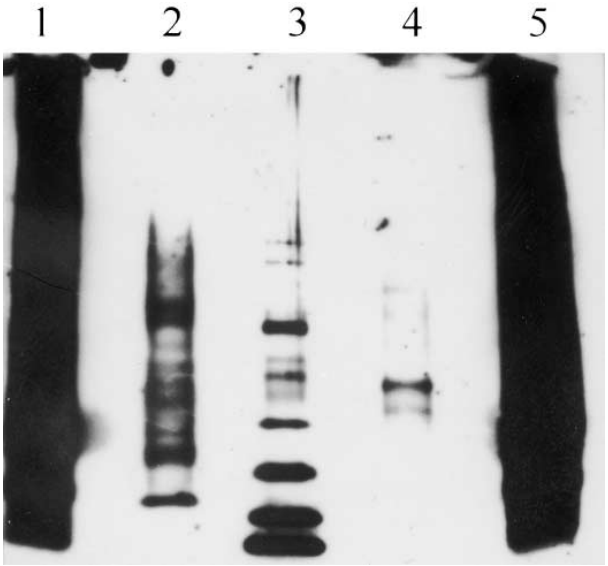

Fig. 4. Detection of GPI-anchored proteins on the surface of *N. tabacum* leaf protoplasts by phase partition assay. Leaf protoplasts were treated as described in Fig. 2. Lane 1 (detergent phase) and lane 2 (aqueous phase) show protein partioning after PtdIns-PLC treatment; lane 4 (aqueous phase) and lane 5 (detergent phase) show protein partioning without PtdIns-PLC treatment. Lane 3 shows molecular weight standards: 116000, 97400, 58100, 39800, 29000, 20100, 14300 and 6500 Da.

er proteins which may be GPI-anchored whereas our study, using biotin labelling, has shown the presence of a number of such proteins in plant cells. In other organisms, such as mammals and yeast, metabolic labelling has also indicated the presence of numerous GPI-anchored proteins [20]. Thus, biotin-based cell surface labelling may be a more successful means for the general detection of such proteins in plants rather than [^{3}H]ethanolamine labelling.

The presence of GPI-anchored proteins in higher plants potentially opens a number of new lines of investigation that have been pursued in other organisms. Particularly interesting areas include pollen/stigma interactions in flowers and adhesion of *Rhizobium* to root hairs in symbioses, as cell surface glycoproteins have been implicated in both processes [21]. It is also possible that a process such as receptor-mediated endocytosis may occur in plants. In this process in mammalian cells, GPI-anchored receptors, such as the folate receptor, are excluded from clathrin-coated pits and accumulate at membrane invaginations called caveolae. Once a ligand is bound, the caveolae close to produce vesicles which transport their contents to the cytosol [22]. The recent demonstration of the presence of smooth pits and vesicles analogous to calveolae in the plasma membrane of plant cells [23], together with our results showing the presence of GPI-anchored proteins, demonstrates that the components necessary for receptor-mediated endocytosis in calveolae do exist in plant cells.

Preliminary data suggest that plant GPI-anchors may contain ceramide in place of diacyglycerol as is the case in yeast [8]. Once the GPI-anchored proteins, identified in the current study, have been purified in sufficient quantity it will be possible to carry out anchor structure analysis to determine if the core structure (ethanolamine-(mannose)$_3$-glucosamine-phosphatidylinositol) is conserved in plants as appears to be the case for all eukaryotes [24].

The presence of GPI-anchored proteins in plants also requires that the machinery for their biosynthesis, within the endoplasmic reticulum, must also be present. The GPI-anchor biosynthetic pathway is thought to involve up to 10 separate steps and genes encoding proteins involved in this process have been cloned from mammals and yeast [25]. One area of future research will involve the cloning and characterisation of homologs to these genes from plant cells.

Acknowledgements: PtdIns-PLC from *Bacillus thuringiensis* was a kind gift of Dr. Martin Low, College of Physicians and Surgeons of Columbia University, New York.

References

[1] Ferguson, M.A.J. and Williams, A.F. (1988) Annu. Rev. Biochem. 57, 285–320.
[2] Takeda, J. and Kinoshita, T. (1995) Trends Biochem. Sci. 20, 367–371.
[3] Brodbeck, U. and Bütikofer, P. (1994) Brazilian J. Med. Biol. Res. 27(2), 369–374.
[4] Low, M.G. (1989) Biochim. Biophys. Acta 988, 427–454.
[5] Lisanti, M.P., Rodriguez-Boulan, E. and Saltiel, A.R. (1990) J. Membr. Biol. 117, 1–10.
[6] Ferguson, M.A.J. (1992) Biochem. Soc. Trans. 20, 243–256.
[7] Stöhr, C., Schuler, F. and Tischner, R. (1995) Planta 196, 284–287.
[8] Morita, N., Nakazato, H., Okuyama, H., Kim, Y. and Thompson Jr., G.A. (1996) Biochim. Biophys. Acta 1290, 53–62.
[9] Gao, J., Lee, J.M. and An, G. (1991) Plant Cell Rep. 10, 533–536.
[10] Robinson, S.P., Edwards, G.E. and Walker, D.A. (1979) in: Plant Organelles, (Reid, E. ed.) pp. 13–24, Ellis Horwood, Chitchester.
[11] Lisanti, M.P., Bivic, A.L., Saltiel, A.R. and Rodriguez-Boulan, E. (1990) J. Membr. Biol. 113, 155–167.
[12] Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250.
[13] Laemmli, U.K. (1970) Nature 227, 680–685.
[14] Birk, H. and Koepsell, H. (1987) Anal. Biochem. 164, 12–22.
[15] Lisanti, M.P. and Rodriguez-Boulan, E. (1990) Trends Biochem. Sci. 15, 113–118.
[16] Bordier, C. (1981) J. Biol. Chem. 256(4), 1604–1607.
[17] Lisanti, M.P., Sargiacomo, M., Graeve, L., Saltiel, A.R. and Rodriguez-Boulan, E. (1988) Proc. Natl. Acad. Sci. USA 85, 9557–9561.
[18] Ilangumaran, S., Robinson, P.J. and Hoessli, D.C. (1996) Trends Cell Biol. 6, 163–167.
[19] Sussman, M.R. (1994) Annu. Rev. Plant Physiol. Plant Mol. Biol. 45, 211–234.
[20] Fankhauser, C., Homans, S.W., Thomas-Oates, J.E., McConville, M.J., Desponds, C., Conzelmann, A. and Ferguson, M.A.J. (1993) J. Biol. Chem. 268(35), 26365–26374.
[21] Larsson, C. and Moller, I.M. (1990) The Plant Plasma Membrane. Spring, Berlin.
[22] Rothberg, K. (1995) Methods Enzymol. 250, 669–679.
[23] Low, P.S. and Chandra, S. (1994) Annu. Rev. Plant Physiol. Plant Mol. Biol. 45, 609–631.
[24] Englund, P.T. (1993) Annu. Rev. Biochem. 62, 121–138.
[25] Kinoshita, T., Miyata, T., Inoue, N. and Takeda, J. (1995) Methods Enzymol. 250, 547–560.